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<b>(54) Title:</b> USE OF BASIC AMINO ACIDS AND DERIVATIVES FOR LOWERING CERAMIDE LEVELS  <b>(57) Abstract</b>  The therapeutic use of basic amino acids, acylated basic amino acids and their pharmacologically acceptable salts is disclosed for the preparation of a medicament for the prophylaxis of illnesses or the therapeutic treatment of cellular disorders accompanied by high levels of ceramide.		

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Use of basic aminoacids and derivatives for  
lowering ceramide levels

The present invention relates to a novel therapeutic use of basic aminoacids, acylated basic aminoacids and their pharmacologically acceptable salts for the prophylaxis of diseases or the therapeutic treatment of cellular disorders accompanied by high levels of ceramide. In particular, the present invention relates to the use of L-carnitine, acyl L-carnitine derivatives and pharmacologically acceptable salts thereof for the prophylaxis of diseases or the therapeutic treatment of cellular disorders accompanied by high levels of ceramide.

Ceramide is the basic molecule for the sphingolipids structure and metabolism thereof. All sphingolipids contain ceramide as main hydrophilic component and originate from ceramide through biosynthesis pathways which mainly modify the 1-hydroxy position thereof. In turn, sphingolipids play an important role in the transduction of the signal across the cellular membrane.

Ceramide plays an important role in the transduction of the signal across the cellular membrane. Molecules able to act upon the intracellular receptors (i.e. calcitriol) or the transmembrane receptors [i.e., gamma interferon (IFN- $\gamma$ ), interleukin-1 (IL-1) and the nerve growth factor (NGF)] hydrolyze sphingomyelin to ceramide. Ceramide activates phosphatases and protein kinases and, from a biological point of view, induces cellular apoptosis, growth and cell differentiation, modulates the expression of cyclooxygenases and phospholipases and the activation of kB nuclear factors (NFkB) [Kuno, K. et al., J. Leukoc. Biol., 56(5): 542-7; Cifone, M. G. et al., J. Exp. Med., 180(4): 1547-52; Kolesnick R., Mol. Chem. Neuropathol., 21(2-3): 287-97; Jarvis, W. D. et al., Proc. Natl. Acad. Sci. U.S.A., 91(1): 73-7; Obeid, L. M. et al., Science, 259(5102): 1769-71].

It has now been found that variations in the concentration or in the metabolism of ceramide contribute to the pathogenesis of numerous illnesses or contribute to induce metabolic cellular disorders. Unfortunately, to date, there exist no methods to reduce ceramide levels *in vivo*.

Accordingly, one object of this invention is to provide a novel use of basic aminoacids, acylated basic aminoacids and their pharmacologically acceptable salt for reducing ceramide levels *in vivo*.

A second object of the present invention is to provide a novel use of basic aminoacids, acylated basic aminoacids and their pharmacologically acceptable salt for the prophylaxis of diseases or the therapeutic treatment of cellular disorders accompanied by high levels of ceramide.

It has in fact been found that administration of high doses of basic aminoacids, low molecular weight basic compounds or acyl derivatives thereof and pharmacologically acceptable salts thereof reduces ceramide levels and such compounds can thus be used for the treatment of diseases characterized by high levels of ceramide.

In particular, it has been found that basic aminoacids such as arginine, lysine, histidine, ornithine, and carnitine or acyl derivatives thereof and pharmacologically acceptable salts thereof can be used for the treatment of diseases characterized by high levels of ceramide.

In accordance with the invention, a novel use of basic aminoacids, basic aminoacid acyl derivatives and pharmacologically acceptable salts thereof is provided for lowering the levels of ceramide *in vivo*.

Suitable aminoacids include any aminoacid with a basic charge such as arginine, lysine, histidine, ornithine and carnitine. These compounds are commercially available. Preferably, L-aminoacids are used. More preferably, carnitine is used. These compounds can be used as free aminoacids or as pharmaceutically acceptable salts.

Acyl derivatives of basic aminoacids can also be used in the present invention. C<sub>2-6</sub> acyl aminoacids which are linear or branched can be used. These acids are well known to the pharmacologists and to the skilled of pharmaceutical technique. Particularly preferred acyl groups are acetyl, propionyl, butyryl, valeryl and isovaleryl.

Suitable pharmaceutical salts can be formed between the above basic aminoacids and any conventional anion such as chloride, bromide, iodide or an acid aspartate such as aspartate, an acid citrate such as citrate, an acid tartrate such as tartrate, an acid phosphate such as phosphate, an acid fumarate, a glycerophosphate such as glucophosphate, acid lactate, acid maleate, orotate; acid oxalate, particularly oxalic acid; a sulfate, particularly preferably sulfate, trichloroacetate, trifluoroacetate and methanesulfonate.

Examples of illnesses or disorders characterized by elevated levels of ceramide include inflammatory bowel diseases, diffuse intravascular coagulation, fever, protein catabolism and/or lipid depletion, hepatosplenomegaly associated with inflammatory or metabolic liver diseases, endo-myocarditis, endothelial cells and leucocytes activation, capillary thrombosis, meningo-encephalitis due to infectious agents, organ transplantation, rheumatoid arthritis and connective tissue diseases, and autoimmune diseases, hyperthyroidism, damages by radiations and/or chemotherapy agents and chronic fatigue syndrome.

Since the use of some drugs can also induce high levels of ceramide, the present invention also contemplates decreasing ceramide levels in patients treated with such a drug. For example, a basic aminoacid in accordance with the present invention can be coadministered with corticosteroids (such as dexamethasone), anti-inflammatory (such as indomethacin), antiviral (such as interferon), immunosuppressants (such as cyclosporin), chemotherapy agents (such as adriamycin), immunopotentiants (such as immunoglobulins and vaccines) and endocrinological agents (such as metimazole) to prevent increased levels of ceramide.

Normal levels of ceramides in healthy patients depend on the age, size and weight of the individual, but are in general within the range of from 5 to 50 picomoles/ $10^6$  cells (preferably, lymphocytes of peripheral blood). Levels higher than 50 picomoles/ $10^6$  cells are regarded as high levels. The use of basic aminoacids, acylated basic aminoacids and pharmacologically acceptable salts thereof of the present invention reduce such high levels by at least 25%.

In general, the basic aminoacids are administered in accordance with the present invention in concentrations which reduce ceramide levels by at least 25%. Suitably, this result is achieved by administering 50 mg to approximately 15 g/day of basic aminoacids by oral or parenteral route. Preferably, high levels of these basic aminoacids should be administered, i.e., >1 g per day, >2 g per day; particularly preferably, 4-10 g per day.

Monitoring ceramide levels can be conducted either by directly monitoring ceramide levels in a cell (such as a lymphocyte) or by indirectly monitoring the concentrations of a ceramide metabolite in a cell. Preferably, the patient's ceramide levels are monitored both prior to and following administration of the basic aminoacid in order to assess the amount of reduction. Monitoring can begin any time following administration but suitably is commenced following 3 hours to ensure accurate results. Monitoring can be continued indefinitely.

Ceramide levels can be directly measured by isolating peripheral blood lymphocytes from the patient. Thereafter the cells are centrifuged to eliminate the supernatant, and the lipids are removed from the cell pellet. The organic phase containing the ceramide can be assayed using the "DAG kinase assay" for phosphorylating the ceramide which is then evidenced by autoradiography [Cifone, M. G. et al., J. Exp. Med., 180(4): 1547-52].

Having generally described this invention, a further understanding can be obtained by reference to certain specific examples which are

provided herein for purposes or illustration only and are not intended to be limiting unless otherwise specified.

### Example 1

Peripheral blood lymphocytes were isolated according to the classical methodologies. The cells were incubated with L-carnitine (200 mcg/ml) or with isovaleryl L-carnitine for 30 min at 37°C, and afterwards with an anti-Fas monoclonal antibody for another 30 min. The cells were then centrifuged, eliminating the supernatant, and the cell pellet was delipidized. The organic phase (containing the ceramide) was assayed in the "DAG kinase assay" for phosphorylating ceramide which subsequently was evidenced by autoradiography.

The results are indicated below in Table 1.

Table 1

	Ceramide (picomoles per 10 <sup>6</sup> cells)
Control	20
Control + anti-Fas antibody	81.6
Control + anti-Fas antibody + L-carnitine (100 mcg/ml)	7.3
Control + anti-Fas antibody + isovaleryl L-carnitine (50 mcg/ml)	8.6
Control + anti-Fas antibody + isovaleryl L-carnitine (100 mcg/ml)	7.3

It is known that the cells suitably stimulated (i.e. with Fas-L, interleukin-1, etc.) generate ceramide. An anti-Fas antibody was employed to increase the production of ceramide from a basal value (20 picomoles per 10<sup>6</sup> cells) to 81.6 picomoles per 10<sup>6</sup> cells.

L-carnitine and isovaleryl L-carnitine are thus shown to inhibit the synthesis of ceramide *in vitro*.

### Example 2

Two patients affected by symptomatic neuro-myopathy (chronic fatigue syndrome) were treated with 3 g per day of L-carnitine by oral route through two months.

Ceramide was measured in the muscles before and after the administration.

The results are indicated below in Table 2.

Table 2

	Ceramide pre-treatment (picomoles per mg of proteins)	Ceramide post-treatment (picomoles per mg of proteins)
Patient 1	76	28
Patient 2	142	46

### Example 3

Four patients affected by hyperthyroidism who had been treated with metimazole (15 mg by oral route, daily) for more than eight months, were treated for 4 weeks with 8 g per day L-carnitine by oral route.

The lymphocyte-associated ceramide was determined before and after the treatment.

The results are shown in the Table 3 which follows.



Table 3

	Ceramide pre-treatment (picomoles/10 <sup>6</sup> cells)	Ceramide post-treatment (picomoles/10 <sup>6</sup> cells)
Patient 1	73	26
Patient 2	45	27
Patient 3	111	36
Patient 4	69	18

Example 4

Three patients with hepato-splenomegaly due to viral hepatitis of C type were treated with 4 g of L-carnitine bolus by intravenous route.

The lymphocyte-associated ceramide was determined before and after 3 and 48 hours following the infusion.

The results are shown in the Table 4 which follows.

Table 4

	Patient 1 Ceramide (picomoles/10 <sup>6</sup> cells)	Patient 2 Ceramide (picomoles/10 <sup>6</sup> cells)	Patient 3 Ceramide (picomoles/10 <sup>6</sup> cells)
Pre-treatment	65	77	79
After 3 hrs	12	32	24
After 48 hrs	31	21	23

It is apparent that the administration of a bolus of L-carnitine inhibited the increase of ceramide levels already after 3 hours from the infusion. The effect remains at least for two days.

### Example 5

Four patients affected by protein catabolism and lipidic depletion as a consequence of tubercular infection were treated for two weeks with 8 g per day L-carnitine by parenteral route.

The peripheral blood lymphocyte-associated ceramide was determined before and after the treatment.

The results are shown in the Table 5 which follows.

Table 5

	Ceramide pre-treatment (picomoles/ $10^6$ cells)	Ceramide post-treatment (picomoles/ $10^6$ cells)
Patient 1	127	59
Patient 2	265	77
Patient 3	301	152
Patient 4	78	54

Having now fully described the invention, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the invention as claimed herein.

### Claims

1. Use of basic aminoacids, acylated basic aminoacids and pharmacologically acceptable salts thereof for producing a medicament for the prophylaxis of diseases or the therapeutic treatment of cellular disorders accompanied by high levels of ceramide.
2. The use of claim 1 wherein the illnesses or disorders characterized by high levels of ceramide comprise inflammatory bowel diseases, diffuse intravascular coagulation, fever, protein catabolism and/or lipid depletion, hepato-splenomegaly associated with inflammatory or metabolic liver diseases, endo-myocarditis, endothelial cells and leucocytes activation, capillary thrombosis, meningo-encephalitis due to infectious agents, organ transplantation, rheumatoid arthritis and connective tissue diseases, and autoimmune diseases, hyperthyroidism, damages by radiations and/or chemotherapy agents and chronic fatigue syndrome.
3. The use of claim 1 or 2 wherein the basic aminoacids, acylated basic aminoacids or pharmacologically acceptable salts thereof are coadministered with corticosteroids, anti-inflammatory, antiviral, immunosuppressants, cytostats, immunopotentiants and endocrinological agents.
4. The use of any of the preceding claims wherein the basic aminoacid is arginine, lysine, hystidine, ornithine or carnitine.
5. The use of claim 4 wherein the basic aminoacid is L-carnitine.
6. The use of any of claim 1-3 wherein the acyl group of said basic aminoacid is selected from acetyl, propionyl, butyryl, valeryl and isovaleryl.
7. The use of claim 6 wherein said acylated basic aminoacid is isovaleryl L-carnitine.

8. The use of any of claims 1-3 wherein the anion of said pharmacologically acceptable salt is selected from the group consisting of chloride, bromide, iodide, acid aspartate, acid citrate, acid tartrate, acid phosphate, acid fumarate, glycoposphate, acid lactate, acid maleate, orotate, acid oxalate and sulphate.

9. The use of claim 8 wherein said anion is sulphate, trichloroacetate, trifluoroacetate or methanesulfonate.

10. The use of claim 1 wherein the daily dose administered orally or parenterally is from 50 mg to 15 g.

11. The use of claim 1 wherein the daily dose administered orally or parenterally is from 4-10 g.

12. An orally or parenterally administrable pharmaceutical composition for the prophylaxis of illnesses or therapeutic treatment of cellular disorders accompanied by high levels of ceramide, which comprises as active ingredient an amount of basic aminoacids, acylated basic aminoacids or pharmacologically acceptable salts thereof effective for reducing the levels of ceramide, and at least a pharmacologically acceptable excipient therefor.

13. The orally or parenterally administrable pharmaceutical composition of claim 12 wherein the illnesses or disorders characterized by high levels of ceramide comprise inflammatory bowel diseases, diffuse intravascular coagulation, fever, protein catabolism and/or lipid depletion, hepato-splenomegaly associated with inflammatory or metabolic liver diseases, endo-myocarditis, endothelial cells and leucocytes activation, capillary thrombosis, meningo-encephalitis due to infectious agents, organ transplantation, rheumatoid arthritis and connective tissue diseases, and autoimmune diseases, hyperthyroidism, damages by radiations and/or chemotherapy agents and chronic fatigue syndrome.

14. The composition of claim 12 or 13 suitable for orally or parenterally administering from 50 mg to 15 g/day of basic aminoacid.

15. The composition of claim 12 or 13 suitable for orally or parenterally administering from 4 g to 10 g/day of basic aminoacid.

